(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
13 February 2003 (13.02.2003)

PCT

(10) International Publication Number
WO 03/012142 A1

(51) International Patent Classification[7]: C12Q 1/68

(21) International Application Number: PCT/NL02/00519

(22) International Filing Date: 31 July 2002 (31.07.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0118758.2 1 August 2001 (01.08.2001) GB

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 03/012142 A1

(54) Title: DETECTION OF NUCLEIC ACIDS BY REAL-TIME PCR USING CHIMERIC RNA-DNA PRIMERS

(57) Abstract: The present invention relates to methods for detection and quantification of target sequences in nucleic acid sample using real-time PCR. The method advantageously employs a chimeric RNA:DNA primer that introduces a unique (universal) detection sequence during a first gene-specific primer-extension reaction. The introduction of this unique detection sequence allows to use a single (universal) fluorescently labelled detection probe for detection and quantification of multiple different target sequence specific amplification products in real-time polymerase chain reactions. The method may be applied for detection and quantification of both DNA and RNA target sequences and is preferably used for quantification of specific mRNA's. By introducing a primer ligation step the method is adapted for genome wide transcriptome analysis. The method can be adapted for differential gene expression analysis in a single PCR reaction, allowing simultaneous and comparative analysis of specific gene-expression levels in samples obtained from cells grown under different conditions.

Detection of nucleic acids by real-time PCR using chimeric RNA-DNA primers

Field of the invention

The present invention relates to methods for detection and quantification of a target nucleic acid by means of real-time PCR. The methods involve the use of a chimeric DNA-RNA oligonucleotide primer to introduce a unique and universal template sequence that allows to use a single universal fluorescent detection probe for the quantification of multiple different target nucleic acids.

Background of the invention

Real time PCR amplification allows the quantitative detection of the logarithmically increasing amount of PCR product in a specific PCR reaction. Three main real-time PCR machines are currently on the market: (1) The light cycler, developed by ROCHE (http://www.biochem.roche.com/lightcycler/) (Wittwer et al. 1989, Nucleic Acids Res. 17: 4353-7; Wittwer et al., 1997, Biotechniques. 22: 176-81), (2) the Taqman (commercialised by Perkin Elmer-Applied Biosystems (http://www.appliedbiosystems.com/products/, generating information on the ABI-PRISM 7700, 7900HT, and 5700 machines), and the (3) iCycler commercialised by BIO-RAD (http://www.bio-rad.com/iCycler/) (for an overview of characteristics of the respective machines, the reader is referred to a molecular biology tools website: www.nlv.ch/Molbioltoolsrtpcr.html. (See also Bustin, 2000, J. Mol. Endocrinol. 25: 169-93).

All three technologies depend on a similar detection method that is based on the real-time detection during a PCR amplification, of a fluorescent signal, the strength of which is proportional to the specific PCR product that is amplified. Yet, the molecular basis that underlies the generation of a quantitative fluorescence signal that corresponds to the amount of the PCR product is different in these three technologies. The Light Cycler e.g., can be used with a double strand DNA (dsDNA) fluorophore that specifically interacts with ds-DNA but does not produce a fluorescent signal with single strand DNA. Thus with increasing amounts of ds-DNA, generated through PCR amplification of the template, an increasing level of fluorescence is generated, thereby allowing quantification. A disadvantage of this method is that the generation of the fluorescent signal does not involve any specificity for the nucleotide sequence that is

amplified. As a consequence, any dsDNA molecule in the reaction mixture, including aspecific amplification products, will contribute to the signal, which will result in an overestimation of the specific amplification product.

Other real-time detection techniques do depend on nucleotide sequence specific fluorescence. For the Light Cycler, a hybridisation probe detection system has been set-up that utilises fluorophore energy transfer between two fluorescent groups. Similarly, detection tools have been developed for the Taqman and iCycler machines: SYBR® green (Morrison et al., 1998, Biotechniques 24: 954-8, 960, 962), TaqMan® probes (inter alia DNA-binding dyes, molecular beacons, hydrolysis probes), Molecular Beacons® (Stratagene) (Tyagi and Kramer, 1996, Nat. Biotechnol. 14: 303-8), and others, that are based on variable physical characteristics of the compounds used and generate a quantitative fluorescence signal reflecting the logarithmically increasing DNA duplex molecule during the PCR reaction cycles that is either non-specific or sequence specific.

The real time detection of mRNA's in various crude samples requires a sequence specific approach to avoid false-positive signals and quantification artefacts. Many research groups employ the real-time PCR quantification method for the differential expression profiling of specific genes in specific tissues or in cells grown under different conditions. The latter is clearly illustrated by a number of recent papers in which the real-time reverse transcription PCR technology has been applied for mRNA quantification: Marcucci et al., 2001, Leukemia, 15:1072-80; Brieland et al., 2001, Infect Immun. 69: 5046-55; Efferth et al., 2001, Int. J. Oncol. 19: 367-71; Evans et al., 2001, J. Clin. Endocrinol. Metab. 86: 3097-107; Elmaagacli et al., 2001, Br. J. Haematol. 113: 1072-5; Harness et al., 2001, J. Neurol. Sci. 187: 7-16; Takano et al., 2001, Br. J. Cancer. 2001 85: 102-6; Reid et al., 2001, Proc. Natl. Acad. Sci. U S A. 98: 7552-7; Jeyaseelan et al., 2001, Nucleic Acids Res. 29:E58-8; Harsch et al., 2001, Am. J. Pathol. 158: 1985-90; Faneyte et al., 2001, Int. J. Cancer 93: 114-22; Haufroid et al., 2001, Clin. Chem. 47: 1126-9; Wang et al., 2001, Mol. Vis. 7: 89-94; Kipar et al., Vet. Immunol. Immunopathol. 78: 305-15; Seeger et al., 2001, Cancer Res. 61: 2517-22; Latil et al., 2001 Cancer Res. 61: 1919-26; Wellmann et al., 2001, Clin. Chem. 47: 654-60; Godfrey et al., 2000, J. Mol. Diagn. 2: 84-91; Amabile et al., 2001, Haematologica.86: 252-9; Aerts et al., 2001, Ann. Oncol. 12: 39-46.

A disadvantage of the sequence specific detection methods for real-time PCR is that for each specific target sequence to be quantified, a specific fluorescent detection probes needs to be prepared. There is thus a need in the art for a real time PCR quantification method in which a universal fluorescent detection probe can be used to quantify multiple different target sequences, such as e.g. mRNA's.

Description of the invention

Quantitative detection of target sequences in nucleic acid samples, such as mRNA molecules, can be performed by real-time PCR reactions using a fluorescent detection method that generates an increasing fluorescence signal with an increasing amount of amplification product generated during the PCR reaction. Real-time detection of this increasing fluorescent signal thus allows to quantify the amount of a specific mRNA template present in the original sample. This technology has been well established in the art as is evident from a large number of publications (see above). The detection methods used in real-time PCR are normally based on fluorescently labelled oligonucleotide probes that are complementary to a sequence in the amplification product that is produced in the PCR reaction (see above). Such fluorescent probes are usually based on an internal region in the target sequence that is being quantified, and therefore, the quantitative detection of specific target DNA or RNA molecules (e.g. a gene or its transcript) requires the design, optimisation and synthesis of sequence-specific fluorescent probes for each target sequence to be analysed. The design and synthesis of such sequence-specific probes is a time-consuming and expensive procedure. For this reason, although possible, it is not very attractive to use real-time PCR for (whole genome) transcription analysis since for each individual gene a specific fluorescent probe has to be designed and tested. Thus it is an object of the present invention to provide methods and materials that allow the use of a single fluorescent probe for the quantitative detection of multiple DNA or RNA target sequence. Such methods are based on the use of chimeric RNA:DNA primers.

In a first aspect the present invention therefore relates to a method (see Figure 1) for detecting a target sequence in a nucleic acid sample, wherein the method comprises the steps of:

(a) providing to a nucleic acid sample a chimeric oligonucleotide primer ABC, whereby the chimeric oligonucleotide primer comprises three sections A, B, and C

that are linked in the 5' to 3' direction, whereby

(i) section A consist of DNA and comprises a unique (universal) priming sequence that is not present in the target sequence or in a sequence complementary to the target sequence;

(ii) section B consists of RNA and comprises a unique detection sequence that is different from the priming sequence and that is not present in the target sequence or in a sequence complementary to the target sequence;

(iii) section C consists of DNA and comprises at its 3'-end a sequence that is complementary to a first sequence in the target sequence;

and allowing the chimeric oligonucleotide primer to anneal to the target sequence;

(b) providing to the nucleic acid sample a first template-dependent DNA polymerase and deoxynucleotide-triphosphates and incubating the sample under conditions whereby the DNA polymerase extends from the chimeric oligonucleotide primer a DNA strand that is complementary to the target sequence;

(c) providing to the nucleic acid sample an oligodeoxynucleotide primer XF that comprises at its 3' end a sequence that is identical to a second sequence in the target sequence, whereby the first sequence in the target sequence is located downstream in a 5' to 3' direction from the second sequence in the target sequence, allowing the oligodeoxynucleotide primer XF to anneal to the DNA strand complementary to the target sequence that is obtained in step (b), and incubating the sample under conditions whereby the DNA polymerase extends from the oligodeoxynucleotide primer XF a DNA strand having the sequence of target sequence fused to the sequences complementary to sections A, B and C of the chimeric oligonucleotide primer, whereby optionally a second template-dependent DNA polymerase is provided to the nucleic acid sample;

(d) providing to the nucleic acid sample an endoribonuclease that hydrolyses phosphodiester bonds between ribonucleotides in an RNA:DNA duplex and incubating the sample under conditions whereby the RNA section B of the chimeric primer is hydrolysed so as to produce a gapped duplex DNA molecule;

(e) providing to the nucleic acid sample a third template dependent DNA polymerase having 5' nuclease activity and a detection probe XX, whereby the detection probe comprises the unique detection sequence and whereby the detection probe

comprises a latent fluorophore that becomes fluorescent upon release from the detection probe by nuclease digestion of the probe;

(f) subjecting the nucleic acid sample to at least one amplification cycle under conditions whereby the target sequence is amplified from the oligodeoxynucleotide primer XF and the unique priming sequence of section A of the chimeric primer, and whereby the amplification of target sequence is detected by determining the fluorescence released from the detection probe.

In the nucleic acid sample, the nucleic acids comprising the target may be any nucleic acid of interest. The nucleotide sequence information contained in the sample may be from any source of nucleic acids, including e.g. RNA, polyA$^+$ RNA (mRNA), cDNA, genomic DNA, organellar DNA such as mitochondrial or chloroplast DNA, synthetic nucleic acids, DNA libraries, clone banks or any selection or combinations thereof. The nucleic acids in the nucleic acid sample may be double stranded, single stranded, and double stranded nucleic acids denatured into single stranded DNA. Denaturation of double stranded sequences yields two single stranded fragments one or both of which can be analysed by probes specific for the respective strands. Preferred nucleic acid samples comprise target sequences on RNA, preferably mRNA. The method of the invention may be applied on mRNA target sequences present in total RNA nucleic acid samples without requiring further purification of the mRNA. Alternatively, the mRNA may be purified form the total RNA e.g. by oligo-dT chromatography.

The chimeric oligonucleotide ABC comprises three sections A, B, and C that are covalently linked in the 5' to 3' direction. Section A consists of deoxynucleotides (DNA) and comprises a unique priming sequence. The unique priming sequence preferably is a sequence that is not present in the target sequence or in a sequence complementary to the target sequence. The unique priming sequence preferably is a sequence that is suitable to serve as a primer-binding site for amplification primers in PCR. The length of the priming sequence may vary from 15 to 40, preferably from 18 to 30, more preferably from 20 to 25. A priming sequence preferably is optimised to meet a number of criteria for optimal use as PCR primer, such e.g. the absence of sequences that can form hairpins or other secondary structures. Such optimal priming sequence can be designed using a standard PCR-primer selection program such as Primer Designer version 2.0 (copyright 1990, 1991, Scientific and Educational

software). The priming sequence may comprise the sequence of commercially available universal primers, such as e.g. the M13 primer.

Section B preferably consists of ribonucleotides (RNA). However, throughout this specification, when referred to the RNA-nature of section B it is to be understood that section B may also consist of other specifically degradable nucleic acids. Specifically degradable nucleic acids are herein understood to mean nucleic acids that may be enzymatically, chemically or physically degraded in the sample while the other nucleic acids, usually poly- and oligodeoxynucleotides, remain intact. E.g. section B may consist of PNA that is specifically degradable by peptidases. Section B comprises a unique detection sequence that is different from the priming sequence and that is not present in the target sequence or in a sequence complementary to the target sequence. The length of the priming sequence may vary from 15 to 50, preferably from 20 to 40, more preferably from 25 to 35. Preferably the detection sequence is free of sequences capable of forming hairpins or other secondary structures (see above).

Section C consists of deoxynucleotides (DNA) and comprises at its 3'-end a sequence that is complementary to a first sequence in the target sequence. The first sequence in the target sequence preferably is located at the 3'-end of the target sequence, i.e. at or near the 3'-end of the part of the target sequence that is amplified and detected. It does not necessarily mean at the most 3'-end of the complete target sequence. In fact, the section C sequence that is complementary to a first sequence in the target sequence may be located in the 5'-half of the target sequences, as long as the first sequence in the target sequence is located downstream (in a 5' - 3' direction) from the second sequence in the target sequence, that is identical or corresponds to the sequence at the 3'-end of the primer XF sequence (see below). The length of complementary sequence may vary from 15 to 40, preferably from 18 to 30, more preferably from 20 to 25. The complementary sequence preferably is optimised to meet a number of criteria for optimal use as an extension primer, such e.g. the absence of sequences that can form hairpins or other secondary structures (see above). Taking into account the above criteria, the skilled person will be able to locate an optimal sequence in the target sequence that could serve as a template for designing the complementary sequence.

For optimal performance of the method of the invention in terms of specificity and reliability of detection and quantification, the various above mentioned

oligonucleotide sequences have preferred melting temperatures. A first melting temperature is the melting temperature of the detection sequence in the detection probe XX, a second melting temperature is the melting temperature of the unique priming sequence in section A of the chimeric primer, and a third melting temperature is the melting temperature of the sequence in section C of the chimeric primer that is complementary to the first sequence in the target sequence. Preferably, in the method according to the invention, the first melting temperature is higher than the second melting temperature, and, preferably the second melting temperature is higher than the third melting temperature. More preferably, the first melting temperature is at least 3, 4, 5, 6 or 8°C higher than the second melting temperature, and more preferably, the second melting temperature is at least 3, 4, 5, 6 or 8°C higher than the third melting temperature. Thus, T_m probe XX > T_m primer A > T_m primer C. As an example may be given: T_m probe XX = 65 °C; T_m primer A = 55 °C; and T_m primer C = 50 °C.

Oligonucleotides for use in the present invention, such as the chimeric oligonucleotide primer and oligodeoxynucleotide primers and probes, may be synthesised using methods well known in the art (see e.g. in Sambrook and Russell (2001) "Molecular Cloning: A Laboratory Manual (3rd edition), Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press). Preferably, oligonucleotides may be synthesised on an oligonucleotide synthesiser according to specifications provided by the manufacturer (such as e.g. Applied Bio Systems, CA, USA). The oligoribonucleotide part of the chimeric primer may be synthesised as described in Coljee et al. (Nature Biotechnol. 18: 789-791) or may be obtained from Oligos ETc (Wilsonville, OR, USA).

In step (a) of the method of the invention, the target sequence is brought into contact with the chimeric oligonucleotide primer under hybridising conditions, and the complementary sequence in section C of the chimeric primer is allowed to anneal to its counter part in the target sequence in the sample. Methods and conditions for specific annealing of oligonucleotides to complementary target sequences are well known in the art (see e.g. in Sambrook and Russell (2001) *supra*). Usually, after mixing of the oligonucleotide probes and target sequences the nucleic acids are denatured by incubation for a short period of time (e.g. 30 seconds to 5 minutes) in a low salt buffer (e.g. a buffer containing no salts or less salts than the ionic strength equivalent of 10mM NaCl). The sample containing the denatured probes and target sequences is then

allowed to cool to an optimal hybridisation temperature for specific annealing of the probes and target sequences, which usually is about 5°C below the melting temperature of the hybrid between the complementary sequence of the primer and its counter part sequence in the target sequence.

A nucleotide sequence that is considered complementary to another nucleotide sequence may contain a minor amount, i.e. preferably less than 20, 15, 10, 5 or 2%, of mismatches. Alternatively, it may be necessary to compensate for mismatches e.g. by incorporation of so-called universal nucleotides, such as for instance described in EP-A 974 672, incorporated herein by reference. Since the kinetics of annealing of probes to target sequences is concentration dependent, annealing is preferably performed in a small volume, i.e. less than 10 µl. Under these hybridisation conditions, annealing of primers to target sequences usually is fast and may occur within 20 - 30 seconds. Annealing therefore does not need to proceed for more than 1, 2, 5, 10 or 15 minutes, although a longer annealing time may be used as long as the hybridisation temperature is maintained to avoid aspecific annealing. To avoid evaporation during denaturation and annealing, the walls and lids of the reaction chambers (i.e. tubes or microtitre wells) may also be heated to the same temperature as the reaction mixture. In preferred oligonucleotide primers the length of the complementary section is preferably at least 15, 18 or 20 nucleotides and preferably not more than 30, 40, or 50 nucleotides and the primers preferably have a melting temperature of at least 50°C, 55°C or 60°C.

In such instances where the method of the invention is applied on a nucleic acid sample comprising a target sequence that consists of RNA, the first template-dependent DNA polymerase that is used in step (b) is a DNA polymerase that preferably has reverse transcriptase activity and that preferably is free of RNase activity. The first DNA polymerase preferably also lacks RNase H activity. Suitable template-dependent DNA polymerases that lack RNase activity and have reverse transcriptase activity include e.g. the *Thermus thermophilus* (*Tth*) DNA polymerase (Myers and Gelfand, 1991 Biochemistry 30:7661-6; recombinant *Thermus thermophilus* DNA polymerase is available from Applied Biosystems, Foster City, CA, USA), SUPERSCRIPT RNase H$^-$ RT™, SUPERSCRIPT II H$^-$ RT™, and THERMOSCRIPT RNase H$^-$ RT™ (Invitrogen, Carlsbad, CA). Other suitable template-dependent DNA polymerases that may be applied include e.g. the AMV or MLV reverse transcriptases. Preferably, conditions for cDNA synthesis on a target sequence that consists of RNA are chosen so

as to avoid polymerisation on DNA templates, such that only single stranded RNA is used as template for cDNA synthesis. Such conditions may include avoiding melting of DNA duplexes under condition whereby single stranded RNA molecules do melt, e.g. single stranded RNA molecules may be melted at 65°C, whereas melting of DNA duplex molecules of any significant length requires at least 95°C.

In such instances where the method of the invention is applied on a nucleic acid sample comprising a target sequence that consists of DNA, the first template-dependent DNA polymerase (that lacks RNase activity) that is used in step (b) may be any template dependent DNA polymerase capable of primer extension on the DNA template, and that lacks RNase activity in order to avoid degradation of section B of the chimeric primer. Such DNA polymerases include the above-mentioned DNA polymerases with reverse transcriptase activity but may further include e.g. Taq DNA polymerase, T4 DNA polymerase, T7 DNA polymerase, or *E.coli* DNA polymerase Klenow fragment.

In step (c) of the method of the invention, oligodeoxynucleotide primer XF is provided to the nucleic acid sample. Primer XF comprises at its 3' end a sequence that is identical or corresponds to a second sequence in the target sequence. The term "identical or corresponds to a second sequence in the target sequence" is herein understood to mean that the sequence at the 3'-end of the target sequence has sufficient identity with the second sequence in the target sequence to allow efficient and reliable priming of a polymerase extension reaction on a template that is complementary to the target sequence. Similarly, the sequence in primer XF that corresponds to the second sequence in the target sequence will have T's in stead of U's in corresponding positions in case the target sequence consists of ribonucleotides. Preferably the second sequence in the target sequence is located at or near the 5'-end of the part of the target sequence that is amplified and detected. It does not necessarily mean at the most 5'-end of the complete target sequence. Thus, the second sequence in the target sequence (present in primer XF) and the first sequence in the target sequence (having its complement at the 3' end of section C of the chimeric primer) are preferably located on the target sequence with respect to each other such that a detectable fragment may be amplified from a target sequence template, whereby the second sequence is located upstream in the 5' to 3' direction on the target sequence with respect to the first sequence. Preferably, the first and second sequences in the target sequence are chosen such that the amplified

fragment has a length of at least 60, 80 or 100 bp. In order not to compromise the efficiency of the amplification, the length of the amplified fragment preferably is not longer than 1000, 800, 600 or 500 bp.

The length of the sequence at the 3'-end of primer XF that is present as second sequence in the target sequence may vary from 15 to 40, preferably from 18 to 30, more preferably from 20 to 25. The sequence at the 3'-end of primer XF preferably is optimised to meet a number of criteria for optimal use as an extension/amplification primer, such e.g. the absence of sequences that can form hairpins or other secondary structures (see above). Taking into account the above criteria, the skilled person will be able to located an optimal sequence in the target sequence that could serve as a template for designing the complementary sequence.

In a preferred embodiment of the method, a fourth melting temperature is the melting temperature of the sequence in primer XF that is as second sequence in the target sequence, whereby the fourth melting temperature is identical to or differs by no more than 1, 2, 3, 5 or 7°C from the second melting temperature, i.e. the melting temperature of the unique priming sequence in section A of the chimeric primer. Close proximity of the second and fourth melting temperature is preferred for the amplification in step (f).

In step (c) of the method of the invention, primer XF is preferably allowed to anneal to the DNA strand complementary to the target sequence that is obtained in step (b), under conditions described above. Subsequently, the sample is incubated under conditions whereby a DNA polymerase extends from the primer XF a DNA strand having the sequence of target sequence fused to the sequences complementary to sections B and C of the chimeric oligonucleotide primer. The template-dependent DNA polymerase that was added in step (b) may be used for this purpose, or optionally a second aliquot of this DNA polymerase is added or second different template-dependent DNA polymerase that lacks RNase activity is provided to the nucleic acid sample.

In step (d) of the method of the invention, an endoribonuclease that hydrolyses phosphodiester bonds between ribonucleotides in an RNA:DNA duplex is added. Preferably this duplex-dependent endoribonuclease does not degrade single stranded nucleic acids, or double stranded DNA or RNA. The sample is then incubated under conditions whereby the RNA section B of the chimeric primer is hydrolysed so as to

produce a gapped duplex DNA molecule. A preferred duplex-dependent endoribonuclease that does not degrade single stranded nucleic acids, or double stranded DNA or RNA is RNase H. Likewise, if section B of the chimeric primer consists of another specifically degradable nucleic acid, in step (d) of the method conditions are applied that degrade that specifically degradable nucleic acid, while leaving intact the oligo- and polydeoxynucleotides in the sample. E.g. a peptidase may be applied in step (d) to degrade a section B consisting of PNA.

In such instances where the method of the invention is applied on a nucleic acid sample comprising a target sequence that consists of RNA, in step (d), one or more duplex-independent ribonuclease(s) may be provided in addition to the duplex-dependent endoribonuclease, so as to degrade all poly- or oligoribonucleotide (RNA) molecules in the sample. Suitable duplex-independent ribonucleases for this purpose include e.g. RNase ONE™ Ribonuclease (Promega, Madison, WI), an endoribonuclease that cleaves the phosphodiester bonds 3' of A, C, G and U releasing 2'3'-cyclic monophosphate ribonucleotides; RNase A (Promega, Madison, WI), an endoribonuclease which cleaves 3' at pyrimidines; RNase T1 (Promega, Madison, WI), an endoribonuclease which attacks the 3'-phosphate groups of G-residues and cleaves the adjacent 5' phosphate linkage; Ribonuclease T2 (Invitrogen, Carlsbad, CA, USA) is a non-specific endoribonuclease that hydrolyses RNA and shows a preference for phosphodiester bonds on the 3' side of A residues.

In step (e) of the method of the invention, a third template-dependent DNA polymerase that has 5'-nuclease activity is added for amplification of (part of) the target sequence from primers XF and A. In addition, a detection probe XX is added to the sample. Detection probe XX is an oligonucleotide, preferably an oligodeoxynucleotide, that comprises the detection sequence of section B of the chimeric primer. Detection probe XX further comprises a latent fluorophore system that becomes fluorescent upon release from the detection probe by nuclease digestion of the detection probe. During amplification of the strand of the target sequence that is extended from primer A, the 5'-nuclease activity of the polymerase digests the detection probe XX when hybridised to the target sequence as the polymerase proceeds because it is located downstream of primer A. Suitable latent fluorophore systems that may be incorporated into the detection probe XX are well known in the art and include e.g. SYBR green (Qiagen GmbH, Hilden, Germany). Further latent fluorophore systems are described in Foldes-

Papp et al., 2001, J. Biotechnol. 86:203-224; Solinas et al., 2001, Nucleic Acids Res. 29: E96; Li et al., 2002, Nucleic Acids Res. 30: E5; Afonina et al., 2002 Biotechniques 32:940-944 and 946-949; and Kreuzer et al., 2001, Clin Chem. 47: 486-490; and are available from Epoch Biosciences (Bothell, WA, USA; www.epochbio.com) and include the fluorescent dyes Redmond Red™, Yakima Yellow™ and Bothell Blue™, each of which may be used in combination with the Eclipse™ dark quencher as latent fluorophore systems.

In a preferred embodiment of the method of the invention, in step (e) an oligonucleotide primer having the unique priming sequence of section A at its 3'-end is added to supplement the amount of primer A derived from the chimeric primer by digestion of the duplex-dependent endoribonuclease.

In step (f) of the method of the invention, the nucleic acid sample is subject to at least one amplification cycle whereby (part of) the target sequence is amplified from primers XF and A. In the method of the invention, the (part of) target sequence may be amplified to produce a detectable amplified fragment by any suitable nucleic acid amplification method known in the art. Nucleic acid amplification methods usually employ two primers, dNTPs, and a (DNA) polymerase. A preferred method for amplification is PCR. "PCR" or "Polymerase Chain Reaction" is a rapid procedure for in vitro enzymatic amplification of a specific DNA segment. The DNA to be amplified is denatured by heating the sample. In the presence of DNA polymerase and excess deoxynucleotide triphosphates, oligonucleotides that hybridise specifically to the target sequence prime new DNA synthesis. One round of synthesis results in new strands of, in principle and depending on the length of the parental strands, indeterminate length, which, like the parental strands, can hybridise to the primers upon denaturation and annealing. The second cycle of denaturation, annealing and synthesis produces two single-stranded products that together compose a discrete double-stranded product, exactly the length between the primer ends. This discrete product accumulates exponentially with each successive round of amplification. Over the course of about 20 to 30 cycles, many million-fold amplification of the discrete fragment can be achieved. PCR protocols are well known in the art, and are described in standard laboratory textbooks, e.g. Ausubel *et al.*, (1995) Current Protocols in Molecular Biology, John Wiley & Sons, Inc. and Sambrook and Russell (2001) *supra*. An amplification cycle is

herein thus understood to mean one cycle of subsequent denaturation, annealing and synthesis.

The presence or absence of the target sequence in the nucleic acid sample is detected in step (f) of the method of the invention by determining whether or not an increase in fluorescence has occurred after one or more amplification cycles. The (increase in) fluorescence may be monitored during the amplification cycle(s) in a process referred to as "real-time PCR". As described above, suitable machines are available for performing real-time PCR. Such machine are usually capable of performing real-time PCR on a multitude of different samples. In a preferred method the amount of target sequence present in the nucleic acid sample is quantified by measuring the amount of fluorescence released from the detection probe. The amount of target sequence present in the nucleic acid sample may be quantified by determining the amount of amplification cycles required for the fluorescence to reach a given threshold level.

In such instances where the method of the invention is applied on a nucleic acid sample comprising a target sequence that consists of DNA, steps (a) to (c) may be combined into a single step. Preferably in such a method the first and second template-dependent DNA polymerases are the same DNA polymerase that may be added once in step (a). More preferably in such a method the first, second, and third template-dependent DNA polymerases are the same DNA polymerase that may be added once in step (a). Thus in such a method the procedure may be started with both the chimeric primer ABC and gene specific primer XF and performing two cycles of polymerisation to generate the product of step (c), i.e. the product of reaction B in the mRNA detection scheme of Figure 1.

In a further preferred embodiment of the method of the invention, steps (a) and (b) are performed on at least two different nucleic acid samples, each comprising the (same) target sequence, whereby for each nucleic acid sample a chimeric oligonucleotide primer is provided that differs only in the unique detection sequence but has identical sections A and C. In this method the nucleic acid samples are preferably combined for at least steps (e) and (f), and whereby for each unique detection sequence in the combined samples a corresponding detection probe is provided that comprises a latent fluorophore that upon release from the detection probe

provide a fluorescence that can be distinguished from the fluorescence released from the other detection probes in the combined sample.

In a second aspect, the invention relates to a method for the preparation of a chimeric oligonucleotide primer ABC as defined above (see Figure 3). The method preferably comprises the steps of (a) providing:
(i) a first oligodeoxynucleotide C1 that comprises at its 3'-end a sequence that is complementary to a sequence in a target sequence and at its 5'-end a ligation tag;
(ii) a second chimeric oligonucleotide ABd that comprises three sections A, B, and d that are linked in a 5' to 3' direction, whereby section A is an oligodeoxynucleotide that comprises a unique (universal) priming sequence that is not present in the target sequence or in a sequence complementary to the target sequence, section B is an oligoribonucleotide that comprises a unique detection sequence that is different from the priming sequence and that is not present in the target sequence or in a sequence complementary to the target sequence, and section d is an oligonucleotide having at its 3'-end a ligation tag with a deoxynucleotide in the 3'-terminal position; and
(iii) a third helper oligonucleotide that comprises a first sequence that is complementary to the ligation tag of the first oligodeoxynucleotide C1 and a second sequence that is complementary to the ligation tag (in section d) of the second chimeric oligonucleotide ABd, whereby the first sequence is located 5' and immediately adjacent to the second sequence; and, annealing the third helper oligonucleotide to the first oligodeoxynucleotide C1 and the second chimeric oligonucleotide ABd; (b) providing a DNA ligase and incubating under conditions whereby the 5'-end of the first oligodeoxynucleotide C1 is linked in a phosphodiester bond to the 3'-end of the second chimeric oligonucleotide ABd to produce the chimeric oligonucleotide primer ABC; and, (c) recovery of the chimeric oligonucleotide primer ABC. This method advantageously allows to use a single (universal) chimeric oligonucleotide ABd for detection and quantification of multiple different target sequences in the above described methods. Thereby the method allows to avoid having to synthesise a different chimeric oligonucleotide primer ABC for each target sequence to be analysed.

In the (third) helper oligonucleotide the first sequence that is complementary to the ligation tag of the first oligodeoxynucleotide C1 and a second sequence that is complementary to the ligation tag of the second chimeric oligonucleotide ABd, whereby the first sequence is located 5' and adjacent to the second sequence such that

upon annealing of the helper oligonucleotide probes to the first oligodeoxynucleotide C1 and the second chimeric oligonucleotide ABd, the complementary sections in the respective oligonucleotides are adjacent to each other, such that the first oligodeoxynucleotide C1 and the second chimeric oligonucleotide ABd may be ligated together. This will usually mean that the complementary sections in the respective oligonucleotides C1 and ABd are at the extreme ends of the probes, whereby the chimeric oligonucleotide ABd has its complementary section at its 3'-end, and oligodeoxynucleotide C1 has its complementary section at its 5'-end. Thus when the complementary sections of the respective oligonucleotides Abd and C1 are correctly paired to the opposite bases of the helper oligonucleotide, the 5'-end of oligodeoxynucleotide C1 will be immediately adjacent to the 3'-end of the chimeric oligonucleotide ABd, i.e. not leaving a single nucleotide gap between the two respective ends. In this configuration the immediately adjacent ends of the respective oligonucleotides Abd and C1 may be connected in a phosphodiester bond by enzymes such as nucleic acid ligases. The (third) helper oligonucleotide is preferably designed and synthesised such that it cannot serve as a primer in a primer extension reaction. This may be achieved by synthesising the (third) helper oligonucleotide with a blocking modification at the 3'-end such that it cannot be extended by a polymerase. The modification of the 3'-end of the helper oligonucleotide may comprise any functional modification known in the art to block the formation of a phosphodiester bond between the 3'-hydroxyl group of the helper oligonucleotide and a 5'-phosphate group of a nucleotide. As such the modification may comprise the incorporation at the 3'-end of the helper oligonucleotide of any nucleotide, preferably a 2'-deoxynucleotide, in which the 3'-hydroxyl group is absent or so modified that it is not available for the formation of a phosphodiester bond. Alternatively, the 3'-end of the tag may be so modified that the 3'-hydroxyl group is not available for the formation of a phosphodiester bond due to steric hindrance. Another way to prevent polymerase extension from the helper oligonucleotide is to design the sequence of the oligonucleotide such that its melting temperature is well below the melting temperatures of any of the primers used in the extension reaction while ensuring that during the extension reaction the temperature is maintained above the melting temperature of the helper oligonucleotide.

The respective 5'- and 3'-ends of a pair of the oligonucleotides Abd and C1 that are annealed essentially adjacent to the complementary parts of a target sequence are

connected to form a covalent bond by any suitable means known in the art. The ends of the probes may enzymatically connected in a phosphodiester bond by a ligase, preferably a DNA ligase. DNA ligases are enzymes capable of catalysing the formation of a phosphodiester bond between (the ends of) two polynucleotide strands bound at adjacent sites on a complementary strand. DNA ligases usually require ATP (EC 6.5.1.1) or NAD (EC 6.5.1.2) as a cofactor to seal nicks in double stranded DNA. Suitable DNA ligase for use in the present invention are T4 DNA ligase or *E. coli* DNA ligase. Alternatively, chemical autoligation of modified polynucleotide ends may be used to ligate two oligonucleotide probes annealed at adjacent sites on the complementary parts of a target sequence (Xu and Kool, 1999, Nucleic Acid Res. 27: 875-881).

Subsequent to the ligation in step (b), the chimeric oligonucleotide ABC is recovered in step (c). Recovery, preferably include some form of purification of the chimeric oligonucleotide ABC from the other components in the reaction mixture and may be performed by methods well known in the art (see e.g. Sambrook and Russell, 2001, *supra*). In order to facilitate recovery of the ligated chimeric oligonucleotide ABC, the chimeric oligonucleotide ABd preferably comprises an affinity label. This will allow to recover the chimeric oligonucleotide primer ABC in step (c) from the ligation reaction in step (b) using the affinity label, e.g. using affinity chromatography or magnetic beads comprising an affinity ligand. Preferably the affinity label is located at or near the 5'-end of the chimeric oligonucleotide ABd. Various affinity labels for use in this method are available to the skilled person, of which biotin is a preferred example.

In a further aspect, the present invention relates to a "kit" containing elements for use in the methods of the invention. Such a kit may comprise a carrier to receive therein one or more containers, such as tubes or vials. The kit may further comprise unlabeled or labelled oligonucleotides of the invention, e.g. to be used as primers, probes, which may be contained in one or more of the containers. The oligonucleotides may be present in lyophilised form, or in an appropriate buffer. One or more enzymes or reagents for use in restriction, ligation and/or amplification reactions may be contained in one or more of the containers. The enzymes or reagents may be present alone or in admixture, and in lyophilised form or in appropriate buffers. The kit may also contain any other component necessary for carrying out the present invention, such as buffers,

enzymes, pipettes, plates, nucleic acids, nucleoside triphosphates, filter paper, gel materials, and materials for (affinity) purification of oligonucleotides. Such other components for the kits of the invention are known per se.

Preferably the kit is a kit for the preparation of a chimeric oligonucleotide primer ABC as defined above. The kit preferably comprises: (a) a chimeric oligonucleotide ABd as defined above; and, (b) a helper oligonucleotide as defined above. A further preferred kit is a kit wherein the chimeric oligonucleotide ABd comprises an affinity label. The kit may further preferably comprise reagents for affinity purification using the affinity label, whereby the affinity label preferably is biotin. A preferred enzyme for inclusion in the kit is a DNA ligase. The kit may further comprise one or more components that may be included in the kit selected from the group consisting of: a template-dependent DNA polymerase that lacks RNase activity, a template-dependent DNA polymerase that lacks RNase activity and that has reverse transcriptase activity, template-dependent DNA polymerase that has 5'-nuclease activity, deoxynucleotide-triphosphates, an endoribonuclease that hydrolyses phosphodiester bonds between ribonucleotides in an RNA:DNA duplex, a duplex-independent ribonuclease, an oligonucleotide detection probe comprising a unique detection sequence and comprising a latent fluorophore that becomes fluorescent upon release from the detection probe by nuclease digestion of the probe, and an oligonucleotide primer having a unique priming sequence at its 3'-end is added.

Description of the figures

Figure 1: Schematic representation of reaction schemes involved in quantitative mRNA detection according to the method described. Reverse transcription reaction is performed using total RNA extracts as a template and chimeric RNA-DNA primer ABC. region C of primer ABC anneals to the 3' end of the target mRNA molecule and is extended by reverse transcriptase, reaction A. Gene specific upstream primer XF is added to the mixture and anneals to the cDNA molecule generated in reaction A. One cycle of PCR is performed in which primer XF is extended by the polymerase used and a complete complementary strand of the extended cDNA is formed; extending across the regions B and A of primer ABC. The reaction mixture is then treated with RNase (including RNase H activity), degrading all RNA molecules present in the mixture; original mRNA template and other RNA's, and region B is processed out of duplex molecule generated through reactions A and B, and region B is processed out of residual, excess primer ABC that was present in reaction A. Fluorescent probe XX is added and real-time PCR is performed using the mRNA-derived duplex molecule formed in reactions A, B and C as a template, primers XF and A for amplification (already present in the reaction mixture) and probe XX (that anneals to the target region B_{inv}; and is thus a part of the sequence of region B) for quantification. The number of cycles required for reaching a fluorescence signal that exceeds a chosen threshold reflects the original amount of mRNA that was present in the RNA sample used, provided that measurement is limited to the linear range of PCR amplification (intrinsic requirements of real-time PCR). Thereby quantification of mRNA in a crude RNA sample has been established using a universal fluorescent probe (region B is not included in the gene sequence and is uniquely introduced during reaction A).

Figure 2: Simultaneous measurement of fluorescence signals derived from probe XX and probe XX* allows direct differential measurement of relative expression levels of gene X under conditions that generated sample 1 and conditions that generated sample 2; and corresponding gene X derived mRNA levels '1' and '2', respectively. Technical description, see Figure 1 legend.

Figure 3: Primer ligation method to generate chimeric primers to be applied for differential mRNA detection according to the scheme in figure 2. Gene specific DNA primers C1 for gene C1 (similar for gene C2 and primer C2, etc. etc. eventually for each gene of the chromosome) is extended with a 5'-ligation aiding tag and is 5'-phosphorylated. The chimeric region of the primers (A-B and A*-B*) is provided with a 3'-ligation aiding tag and a 5'-recovery molecule (biotin or other). Sections A and A* may be identical but sections B and B* must be different. Mixing the chimeric primer (separate mixtures for A-B and A*-B*) and the gene specific primer with the complementary oligonucleotide for the two ligation aiding tags targets the DNA ligase to the desired ligation site. After ligation, the ligated chimeric primers are recovered from the ligation mixture utilising the recovery label, according to standard technologies. The recovery product generated can be applied in the differential analysis procedure depicted in Figure 2. For each predicted gene of a genome or a specific subset of genes, these chimeric primers can be generated, using primer ligation steps that utilise universal sequences for AB, AB*, ligation aiding tags and ligation helper oligonucleotides.

Examples

Example 1: Real-time RT-PCR quantification of mRNA molecules primed from chimeric DNA-RNA primers

The example described below, illustrates the application of a chimeric RNA-DNA primer for the quantitative detection of a specific mRNA (see also Figure 1). The quantitative detection of the mRNA transcript from gene X is performed as follows: Chimeric primer ABC (A: 5' end to C: 3' end) contains three regions (A, B, and C), of which region A and C are oligodeoxynucleotides that are linked by an oligoribonucleotide molecule B, thereby creating the chimeric RNA:DNA primer. The sequence of region C in this chimeric oligonucleotide is complementary to a sequence at or near the 3' end of the gene X mRNA of which the transcription level will be examined. Alternatively, the primers may consist of modified DNA or RNA oligomeres, for examples fimers, modified oligonucleotides with proprietary SUC modifications (Fidelity Systems, Gaithersburg, MD, USA; Slesarev et al., 2002, Proc. Natl. Acad. Sci. USA, 99: 4644-4649).

Reverse transcription is performed on a total RNA preparation isolated from cells expressing gene X. The mRNA template and primer ABC are annealed (reaction A in Figure 1) and a reverse transcriptase that lacks RNase H activity is added. Suitable reverse transcriptases for this purpose is e.g. the *Thermus thermophilus* (*Tth*) DNA polymerase, a DNA polymerase with intrinsic reverse transcriptase, but no RNase H activity (Myers and Gelfand, 1991 Biochemistry 30:7661-6). Other suitable reverse transcriptases include SUPERSCRIPT RNase H$^-$ RT™, SUPERSCRIPT II H$^-$ RT™, and THERMOSCRIPT RNase H$^-$ RT™ (Invitrogen, Carlsbad, CA). Conditions are chosen to avoid polymerisation on DNA templates such that only single stranded RNA is used as template for cDNA synthesis. Reverse transcription thus generates a gene X cDNA that is tagged at its 5'-end with the RNA and DNA sequences provided by regions B and A of the primer ABC, respectively (the product of reaction A in Figure 1).

Gene X specific upstream primer XF is subsequently added, which is complementary to a part of the gene X cDNA sequence that is more towards the 5'-end of the gene X sequence relative to sequence chosen for the design of the C region of the chimeric primer ABC. A DNA polymerase without RNase H activity is added to the mixture to extend from primer XF. Preferably, the reverse transcriptase used for cDNA synthesis is a DNA polymerase capable of using both RNA and DNA as template such

that a single polymerase may be used for cDNA synthesis and extension from primer XF. More preferably the DNA polymerase is also PCR compatible. Several DNA polymerases that fulfil these criteria have been described and are commercially available, for example *Thermus thermophilus* (Tth) polymerase a DNA polymerase with intrinsic reverse transcriptase, but without RNase H activity (Myers and Gelfand, 1991, *supra*).

A single DNA polymerisation cycle is performed (reaction B of Figure 1) that will generate a primer XF extension product that is extended across the B and A regions of the chimeric primer ABC used in the reverse transcription reaction (product of reaction B, figure 1). A specific polynucleotide duplex molecule is now generated that contains one strand that consists entirely of deoxynucleotides, encompassing the part of the gene X sequence that is confined by the sequences of primer XF and the region C of the chimeric primer ABC, and which is extended by DNA sequences complementary to the sequences of regions B and A of chimeric primer ABC (top strand of the duplex molecule depicted as the product of reaction B in Figure 1). The other strand of the duplex is a DNA-RNA chimeric molecule that is the primer ABC derived reverse transcriptase extension product, which contains a small ribonucleotide region corresponding to the region B of the chimeric primer ABC and for the remainder consists of deoxynucleotides (gene X, region C and region A of primer ABC).

The reaction product of reaction B (mixture of nucleotide molecules) is subsequently treated with deoxyribonuclease-free ribonucleases (DNase-free RNases), or mixtures of such enzymes, that at least include RNase H activity and preferably also further (general) RNase activities (reaction C in Figure 1): Ribonuclease H (Promega, Madison, WI) is an endoribonuclease that hydrolyses the phosphodiester bonds of RNA:DNA hybrids to produce 3'OH and 5'-phosphate-terminated products. It does not degrade single stranded nucleic acids, duplex DNA or double stranded RNA. RNase ONE™ Ribonuclease (Promega, Madison, WI) is an endoribonuclease that cleaves the phosphodiester bonds 3' of A, C, G and U releasing 2'3'-cyclic monophosphate ribonucleotides. RNase A (Promega, Madison, WI) is an endoribonuclease that cleaves 3' at pyrimidines. RNase T1 (Promega, Madison, WI) is an endoribonuclease that attacks the 3'phosphate groups of G-residues and cleaves the adjacent 5' phosphate linkage. Ribonuclease T2 (Invitrogen, Carlsbad, CA, USA) is a non-specific endoribonuclease that hydrolyses RNA and shows a preference for phosphodiester

bonds on the 3' side of A residues. This treatment will degrade all polyribonucleotide molecules that were originally present in the reverse transcription mixture. Moreover, due to the RNase H activity the nucleotide-duplex product of reaction B will be processed, removing the RNA-region B from the originally chimeric primer ABC sequence, thereby releasing primer A and target region B_{inv} for the fluorescent probe (probe XX) that is complementary to part of the RNA sequence of the B-region of the chimeric primer ABC (reaction product of reaction C in figure 1). Moreover, reaction C will also release primer A and C and degrade part B from all residual chimeric primer ABC that has not been used in reaction A.

The last step of the procedure is a real-time PCR reaction that uses a detection probe XX for PCR product detection, and primers A and XF as PCR primers, both of which are already present in the reaction mixture (reaction D in figure 1). The number of cycles required for reaching a fluorescence signal that exceeds a chosen threshold reflects the original amount of mRNA that was present in the RNA sample used, provided that measurement is limited to the linear range of PCR amplification (intrinsic requirements of real-time PCR).

Example 2: Differential gene expression analysis using chimeric primer based real-time RT-PCR

The method described in Example 1 allows quantification of a specific mRNA molecule in a crude RNA extract from cells grown under a given condition. The adaptation of the technology described in Example 2 allows the differential analysis of gene expression for a specific gene under various conditions simultaneously. The limit of the number of conditions that can be analysed simultaneously is dominated by the limitations in measuring different fluorescence signals simultaneously in one reaction. Systems that generate various light signals that are based on fluorescence and intramolecular energy transfer might allow the increase of the number of different signals that can simultaneously be measured: For example Biorad instruments has launched an optical module that fits their standard thermal cycler and transforms it into a real-time RT-PCR system. This instrument is capable of generating and detecting a wide range of excitation frequencies. By using this instrument it is possible to monitor at least four different fluorescent reporters at any one time (Bustin, *supra*). Multiplex RT PCR can also be applied with the Taqman system (Sharma et al., 1999, Mol. Cell

Probes. 13: 291-302; Shin et al., 1999, J. Clin. Microbiol. 37: 165-70) or by using Molecular Beacons (Tyagi et al., 2000, Nat. Biotechnol. 18: 1191-6). By designing two different chimeric primers ABC in which the regions A and C are identical but the region B is different, one can analyse the level of a specific mRNA in two different samples simultaneously (see description below). When these two RNA samples are obtained from cells grown under different conditions this method allows mRNA specific differential expression analysis. As stated above, the number of samples that can be analysed simultaneously can be increased to the limitation of the number of different fluorescence signals that can be measured simultaneously by the real-time PCR machine and corresponding detection techniques.

Two RNA samples are isolated (sample 1 and sample 2) from cells grown under different conditions. Two chimeric primers ABC are designed for the differential (sample 1 relative to sample 2) quantification of mRNA derived from gene X. Both primers contain identical sequences in region C, allowing equal efficiency in the reverse transcription reaction, and region A. (reaction A in Figure 2). The sequences of region B (RNA) of the chimeric primers used is different. Primers and primer regions are designed in such a way that cross-hybridisation or cross-annealing are excluded under the reaction conditions used in later steps of the procedure. Primers are designated primer ABC and primer AB*C. The two RNA samples are separately used in a reverse transcription reaction identical to reaction A in figure 1 (schematic view: reaction A of Figure 2). Subsequently, the reaction product mixtures are mixed and treated according to reactions B, C and D of the scheme described for the quantification of a single mRNA template (Figure 1). The only adaptation is that for reaction D two specific fluorescent probes that have different excitation and emission wavelengths (allows simultaneous measurement in a mixed sample) are added that have sequences based on the sequences of regions B and B* of the chimeric primers. These probes are designated probe XX and probe XX*, targeting B_{inv} and B^*_{inv} sequences, respectively. The procedure described here is schematically represented in Figure 2.

Alternative to what is stated above it is also possible to analyse different target RNA levels simultaneously. In this case the region C of chimeric primer ABC as well as the region B are different, thereby generating mRNA specific reverse transcription products that can subsequently be detected in a real time PCR reaction in which also the corresponding (different) upstream primers XF are added.

Alternative to what is stated above it also possible to analyse different target RNA levels or specific mRNA molecules originating from crude RNA extracts from cells grown under a certain condition simultaneously by using multiple tubes and instrumentation that allows simultaneously scanning of samples. For example from Biorad Instruments apparatuses are available that can scan up to 96 samples simultaneously.

When necessary, or when no identical amounts of target RNA are isolated, normalisation required due to variation in amount of starting material between samples can be done by amplification of a reference RNA molecule that is expressed at constant level, simultaneously with the target RNA. The reference RNA serves as an internal standard against which the other RNA's may be normalised.

Example 3: Differential gene expression analysis using chimeric primer based real-time RT-PCR; adaptation towards genomics; multiple targets

The differential gene expression analysis described above can be applied in a targeted approach, aiming at the quantitative analysis of the transcription level of a specific gene. To broaden the application territory of the chimeric RNA-DNA primer approach, the primer-length (relatively long primers are required in the chimeric primer approaches described above) will have to be reduced. A solution for this problem can be found in a primer ligation step. Moreover, inclusion of a ligation step also allows the design of chimeric primer approaches towards whole genome transcriptome analysis. Primer ligation encompasses a linkage of the chimeric part of the primer (similar to regions A and B in the strategies described above), which is linked to the gene-specific region of the chimeric primer (similar to the region C in the approaches described above). To facilitate such a primer ligation-procedure, both primers have to be extended with linker sequences that can physically be brought together by a third oligonucleotide complementary to the extension of the chimeric part of the primer and the gene-specific part of the primer (see Figure 3.)

Whole genome approaches can be achieved by the separate ligation of gene-specific primers for each predicted gene within the genome (C1, C2, C3, C4 etc.) of interest to a set of chimeric primer regions that differ in sequence (A-B, A-B* and/or A-B**). The resulting ligated primers will have to be re-isolated from the ligation mixture, which can be performed by using different techniques aiming to specifically

isolate the ligated primers. For example release of the ligation aiding primer from the ligated primer by heating followed by affinity isolation based on an 5'-labelling of the chimeric region of the primer (for example biotin). Alternatively, the ligation aiding primers may be developed in such a way that they cannot interfere in the subsequent reverse transcription or polymerase chain reaction for example by modification of the 5′-end and 3′-end or by developing the ligation aiding primer with a very low annealing temperature preventing annealing to nucleotides during reverse transcription or polymerase chain reaction. The resulting chimeric primers can be used in (differential) gene expression or presence analyses according to the examples described in the previous sections.

The described ligation step may be performed before the RT and PCR. Alternatively, the ligation step can also be performed after RT. In this case the RT is performed by using a primer consisting of region C extended with the linker sequence.

Claims

1. A method for detecting a target sequence in a nucleic acid sample, wherein the method comprises the steps of:

(a) providing to a nucleic acid sample a chimeric oligonucleotide primer ABC, whereby the chimeric oligonucleotide primer comprises three sections A, B, and C that are linked in the 5' to 3' direction, whereby

(i) section A consist of DNA and comprises a unique (universal) priming sequence that is not present in the target sequence or in a sequence complementary to the target sequence;

(ii) section B consists of RNA and comprises a unique detection sequence that is different from the priming sequence and that is not present in the target sequence or in a sequence complementary to the target sequence;

(iii) section C consists of DNA and comprises at its 3'-end a sequence that is complementary to a first sequence in the target sequence;

and allowing the chimeric oligonucleotide primer to anneal to the target sequence;

(b) providing to the nucleic acid sample a first template-dependent DNA polymerase and deoxynucleotide-triphosphates and incubating the sample under conditions whereby the DNA polymerase extends from the chimeric oligonucleotide primer a DNA strand that is complementary to the target sequence;

(c) providing to the nucleic acid sample an oligodeoxynucleotide primer XF that comprises at its 3' end a sequence that is identical to a second sequence in the target sequence, whereby the first sequence in the target sequence is located downstream in a 5' to 3' direction from the second sequence in the target sequence, allowing the oligodeoxynucleotide primer XF to anneal to the DNA strand complementary to the target sequence that is obtained in step (b), and incubating the sample under conditions whereby the DNA polymerase extends from the oligodeoxynucleotide primer XF a DNA strand having the sequence of target sequence fused to the sequences complementary to sections A, B and C of the chimeric oligonucleotide primer, whereby optionally a second template-dependent DNA polymerase is provided to the nucleic acid sample;

(d) providing to the nucleic acid sample an endoribonuclease that hydrolyses phosphodiester bonds between ribonucleotides in an RNA:DNA duplex and

incubating the sample under conditions whereby the RNA section B of the chimeric primer is hydrolysed so as to produce a gapped duplex DNA molecule;

(e) providing to the nucleic acid sample a third template dependent DNA polymerase having 5' nuclease activity and a detection probe XX, whereby the detection probe comprises the unique detection sequence and whereby the detection probe comprises a latent fluorophore that becomes fluorescent upon release from the detection probe by nuclease digestion of the probe;

(f) subjecting the nucleic acid sample to at least one amplification cycle under conditions whereby the target sequence is amplified from the oligodeoxynucleotide primer XF and the unique priming sequence of section A of the chimeric primer, and whereby the amplification of target sequence is detected by determining the fluorescence released from the detection probe.

2. A method according to claim 1, whereby the amount of target sequence is quantified by measuring the amount of fluorescence released from the detection probe.

3. A method according to claim 2, whereby the amount of target sequence is quantified by determining the amount of amplification cycles required for the fluorescence to reach a threshold level.

4. A method according to any one of claims 1 - 3, whereby the target sequence consist of RNA and whereby the first template-dependent DNA polymerase is a DNA polymerase that has reverse transcriptase activity.

5. A method according to claim 4, whereby the target sequence is a mRNA or part thereof.

6. A method according to claims 4 or 5, wherein in step (d) a duplex independent ribonuclease is provided in addition to the duplex dependent endoribonuclease.

7. A method according to any one of claims 1 - 3, whereby the target sequence consist of DNA and wherein steps (a) to (c) are combined in a single step.

8. A method according to claim 7, whereby the first and second template-dependent DNA polymerases are the same DNA polymerase.

9. A method according to claim 8, whereby the first, second, and third template-dependent DNA polymerases are the same DNA polymerase.

10. A method according to any of the preceding claims, whereby a first melting temperature is the melting temperature of the detection sequence in the detection probe XX, a second melting temperature is the melting temperature of the unique priming sequence in section A of the chimeric primer, and a third melting temperature is the melting temperature of the sequence in section C of the chimeric primer that is complementary to the first sequence in the target sequence, and whereby the first melting temperature is higher than the second melting temperature, and whereby the second melting temperature is higher than the third melting temperature.

11. A method according to claim 10, whereby the first melting temperature is at least 5°C higher than the second melting temperature, and whereby the second melting temperature is at least 5°C higher than the third melting temperature.

12. A method according to claims 10 or 11, whereby a fourth melting temperature is the melting temperature of the sequence in the oligodeoxynucleotide primer XF that is identical to the second sequence in the target sequence, and whereby the fourth melting temperature differs by no more than 5°C from the second melting temperature.

13. A method according to any of the preceding claims, whereby in step (e) an oligonucleotide primer having the unique priming sequence of section A at its 3'-end is added.

14. A method according to any of the preceding claims, whereby steps (a) and (b) are performed on at least two different nucleic acid samples, each comprising the target sequence, and whereby for each nucleic acid sample a chimeric oligonucleotide primer is provided that differs in the unique detection sequence; whereby the nucleic acid samples are combined for at least steps (e) and (f), and whereby for each unique

detection sequence in the combined samples a corresponding detection probe is provided that comprises a latent fluorophore that upon release from the detection probe provide a fluorescence that can be distinguished from the fluorescence released from the other detection probes in the combined sample.

15. A method for the preparation of a chimeric oligonucleotide primer ABC as defined in claim 1, the method comprising:

(a) providing:

(i) a first oligodeoxynucleotide C1 that comprises at its 3'-end a sequence that is complementary to a sequence in a target sequence and at its 5'-end a ligation tag;

(ii) a second chimeric oligonucleotide ABd that comprises three sections A, B, and d that are linked in a 5' to 3' direction, whereby section A is an oligodeoxynucleotide that comprises a unique priming sequence that is not present in the target sequence or in a sequence complementary to the target sequence, section B is an oligoribonucleotide that comprises a unique detection sequence that is different from the priming sequence and that is not present in the target sequence or in a sequence complementary to the target sequence, and section d is an oligonucleotide having at its 3'-end a ligation tag with a deoxynucleotide in the 3'-terminal position; and

(iii) a third helper oligonucleotide that comprises a first sequence that is complementary to the ligation tag of the first oligodeoxynucleotide C1 and a second sequence that is complementary to the ligation tag of the second chimeric oligonucleotide ABd, whereby the first sequence is located 5' and immediately adjacent to the second sequence; and

annealing the third helper oligonucleotide to the first oligodeoxynucleotide C1 and the second chimeric oligonucleotide ABd;

(b) providing a DNA ligase and incubating under conditions whereby the 5'-end of the first oligodeoxynucleotide C1 is linked in a phosphodiester bond to the 3'-end of the second chimeric oligonucleotide ABd to produce the chimeric oligonucleotide primer ABC; and

(c) recovery of the chimeric oligonucleotide primer ABC.

16. A method according to claim 14, whereby the second chimeric oligonucleotide ABd comprises affinity label and whereby in step (c) the chimeric oligonucleotide primer ABC is recovered from the ligation reaction in step (b) using the affinity label.

17. A method according to claim 15, whereby the affinity label is biotin.

18. A kit for the preparation of a chimeric oligonucleotide primer ABC as defined in claim 1, whereby the kit comprises:

(a) a chimeric oligonucleotide ABd that comprises three sections A, B, and d that are linked in a 5' to 3' direction, whereby section A is an oligodeoxynucleotide that comprises a unique priming sequence that is not present in the target sequence or in a sequence complementary to the target sequence, section B is an oligoribonucleotide that comprises a unique detection sequence that is different from the priming sequence and that is not present in the target sequence or in a sequence complementary to the target sequence, and section d is an oligonucleotide having at its 3'-end a ligation tag with a deoxynucleotide in the 3'-terminal position; and,

(b) a helper oligonucleotide that comprises a first sequence that is complementary to a sequence that may be used as a ligation tag at the 5'-end of a target sequence specific oligodeoxynucleotide primer and a second sequence that is complementary to the ligation tag of the chimeric oligonucleotide ABd, whereby the first sequence is located 5' and immediately adjacent to the second sequence.

19. A kit according to claim 18, whereby the chimeric oligonucleotide ABd comprises an affinity label.

20. A kit according to claim 19, whereby the kit comprises reagents for affinity purification using the affinity label

21. A kit according to claims 19 or 20, whereby the affinity label is biotin.

22. A kit according to any one of claims 18 - 21, whereby the kit comprises a DNA ligase.

23. A kit according to any one of claims 18 - 22, whereby the kit comprises one or more further reagents selected from the group consisting of: a template-dependent DNA polymerase that lacks RNase activity, a template-dependent DNA polymerase that lacks RNase activity and that has reverse transcriptase activity, template-dependent DNA polymerase that has 5'-nuclease activity, deoxynucleotide-triphosphates, an endoribonuclease that hydrolyses phosphodiester bonds between ribonucleotides in an RNA:DNA duplex, a duplex-independent ribonuclease, an oligonucleotide detection probe comprising a unique detection sequence and comprising a latent fluorophore that becomes fluorescent upon release from the detection probe by nuclease digestion of the probe, and an oligonucleotide primer having a unique priming sequence at its 3'-end is added.

Fig 1 (1)

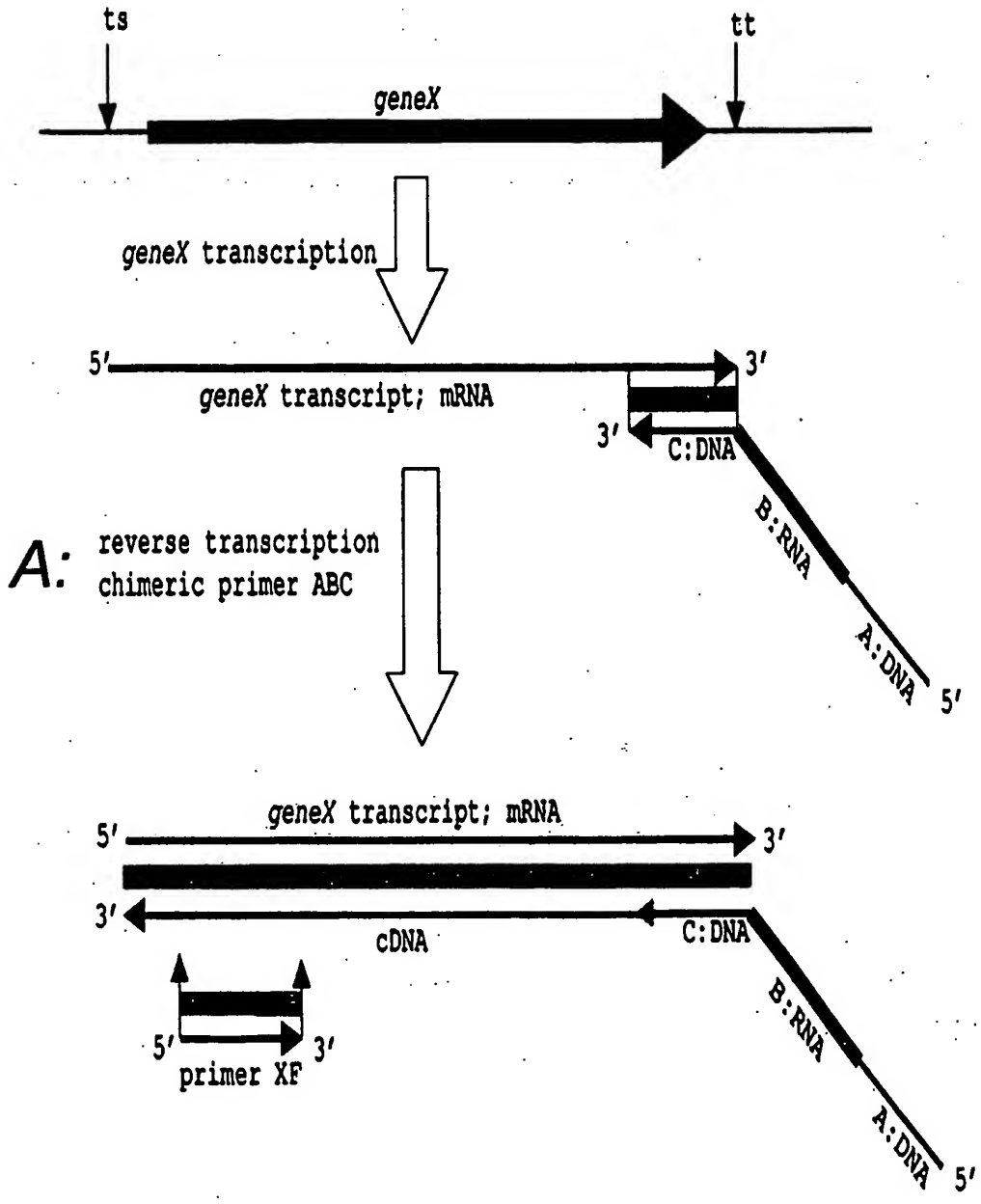

ts
tt
geneX
geneX transcription
5′
3′
geneX transcript; mRNA
3′
C:DNA
B:RNA
A:DNA
5′
A:
reverse transcription
chimeric primer ABC
geneX transcript; mRNA
5′
3′
3′
cDNA
C:DNA
B:RNA
A:DNA
5′
5′
3′
primer XF

Fig 1 (2)

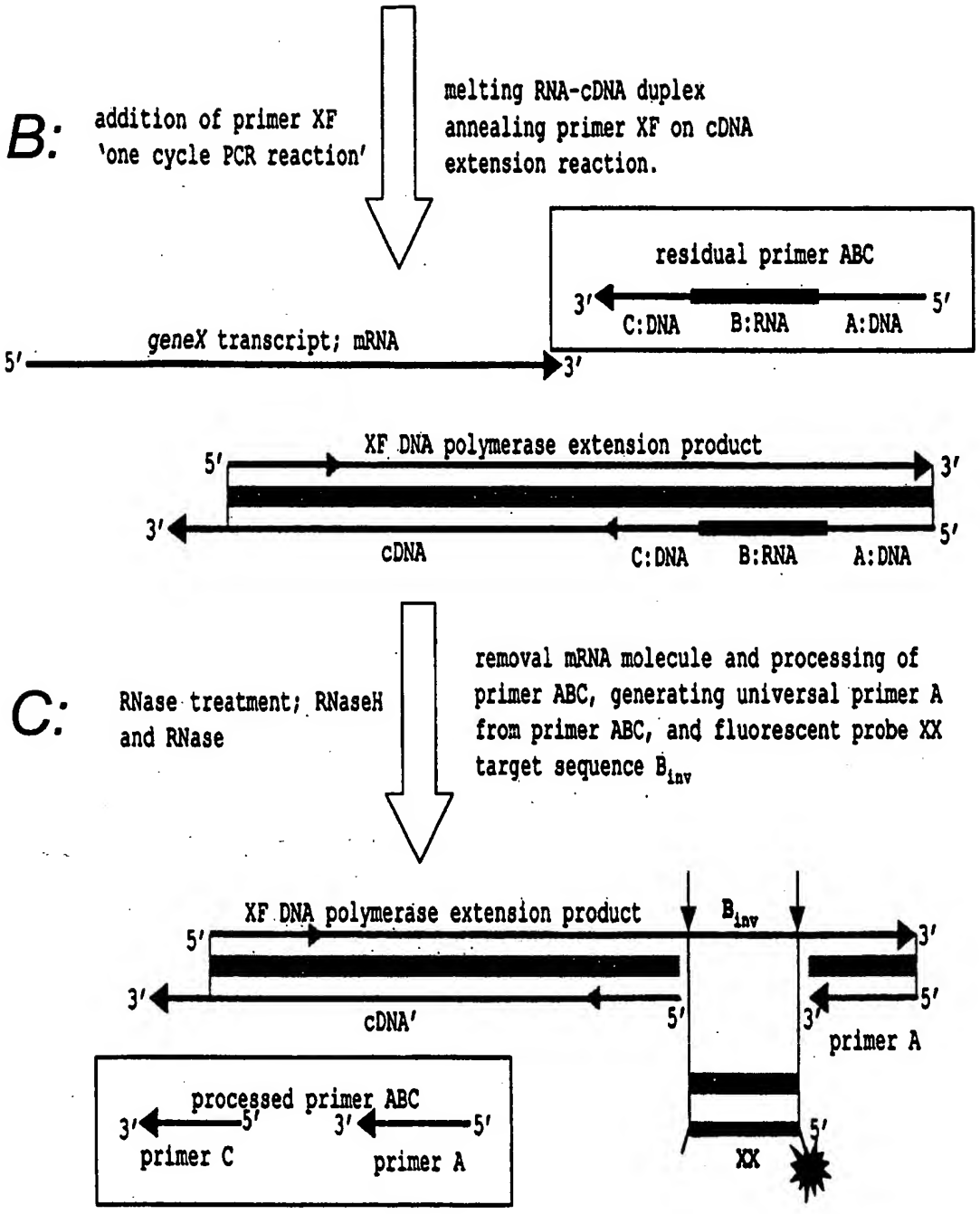

B:
addition of primer XF
'one cycle PCR reaction'
melting RNA-cDNA duplex
annealing primer XF on cDNA
extension reaction.
residual primer ABC
3'
5'
C:DNA
B:RNA
A:DNA
geneX transcript; mRNA
5'
3'
XF DNA polymerase extension product
5'
3'
3'
5'
cDNA
C:DNA
B:RNA
A:DNA
C:
RNase treatment; RNaseH
and RNase
removal mRNA molecule and processing of
primer ABC, generating universal primer A
from primer ABC, and fluorescent probe XX
target sequence B inv
XF DNA polymerase extension product
B inv
5'
3'
3'
5'
cDNA'
5'
3'
5'
primer A
processed primer ABC
3'
5'
3'
5'
primer C
primer A
5'
XX

Fig 1 (3)

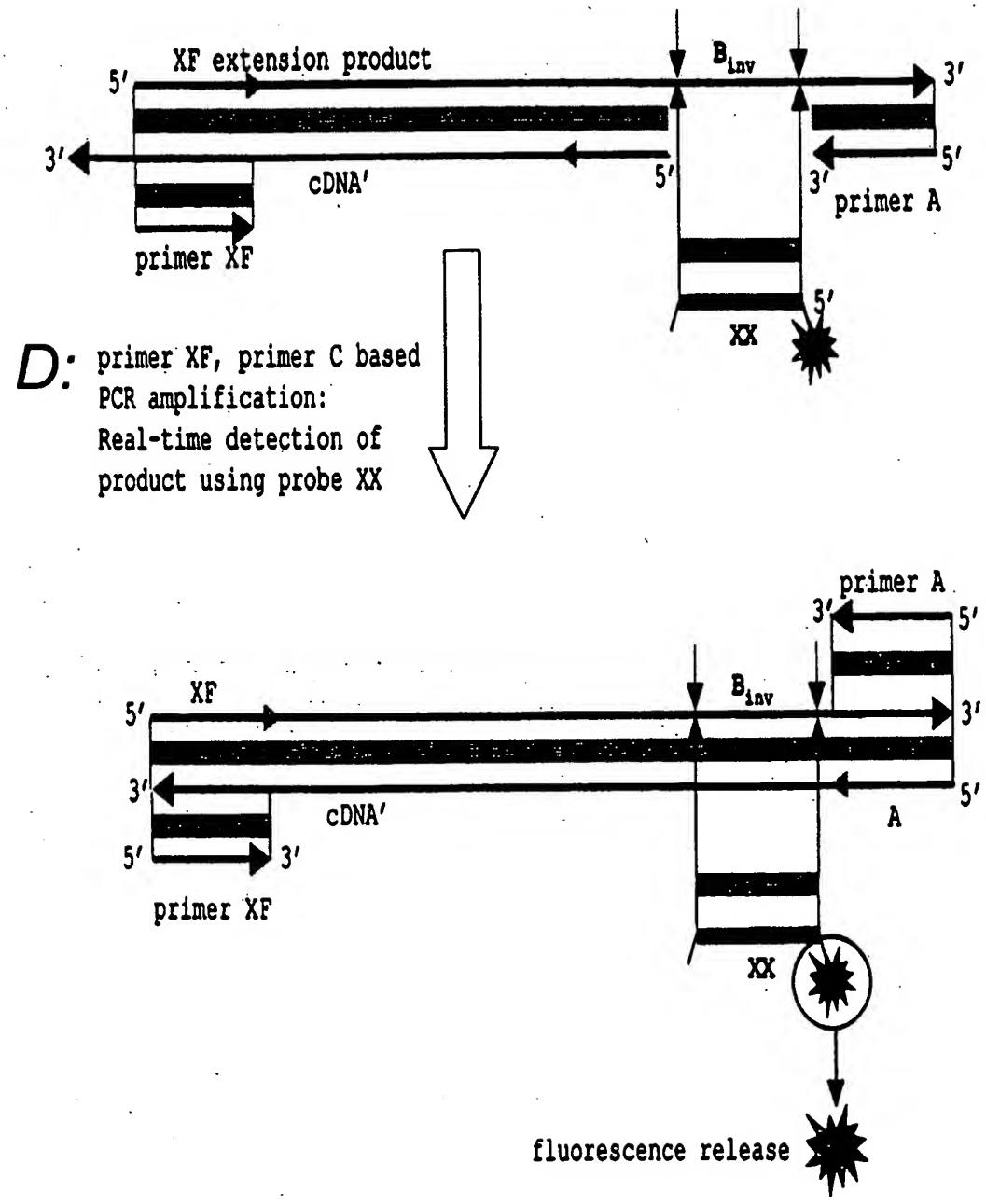

XF extension product
B_{inv}
5′
3′
3′
5′
cDNA′
5′
3′
5′
primer A
primer XF
5′
XX
D: primer XF, primer C based PCR amplification: Real-time detection of product using probe XX
primer A
3′
5′
XF
B_{inv}
5′
3′
3′
5′
cDNA′
A
5′
3′
primer XF
XX
fluorescence release

Fig 2(1)

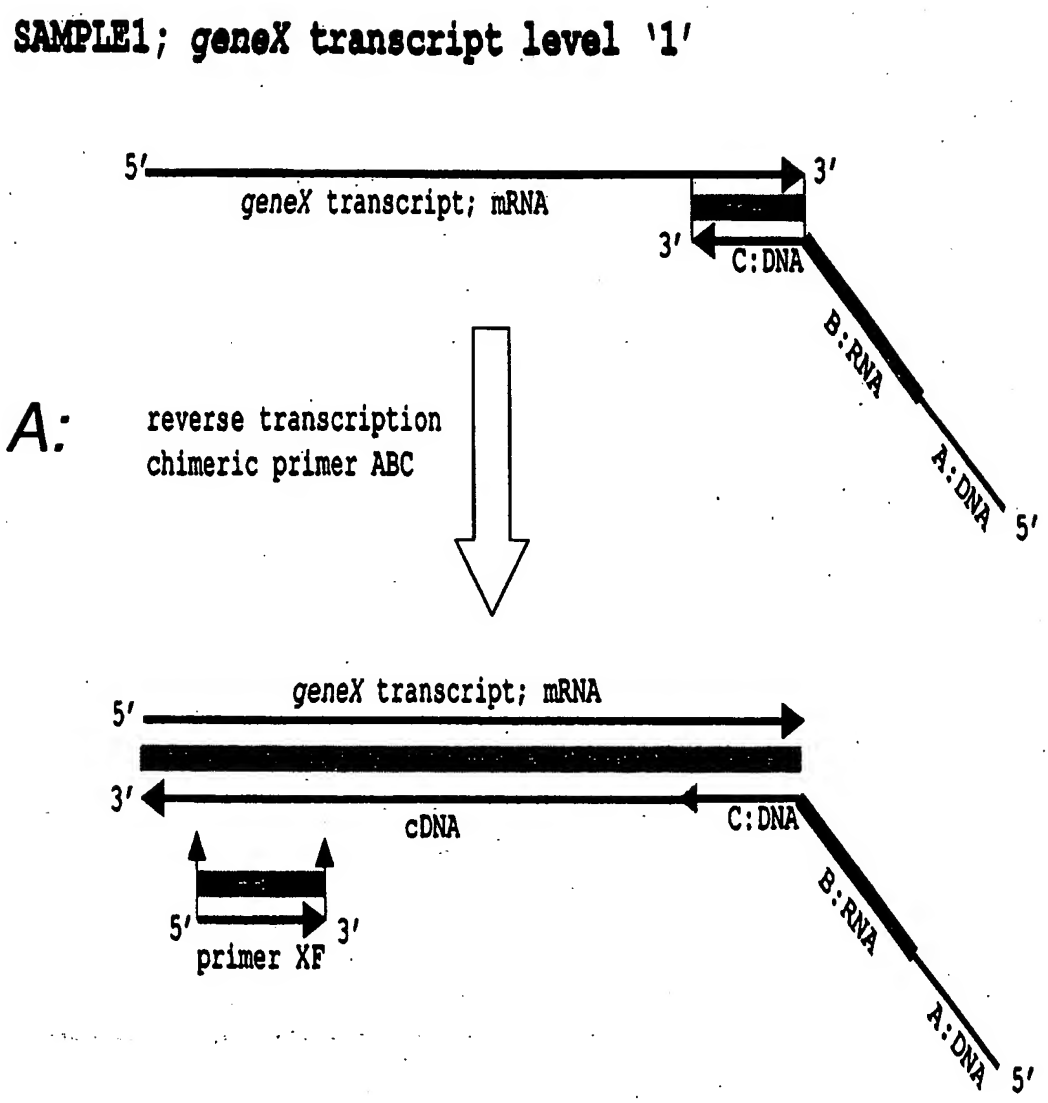

SAMPLE1; geneX transcript level '1'
5′
geneX transcript; mRNA
3′
3′
C:DNA
B:RNA
A:DNA
5′
A:
reverse transcription
chimeric primer ABC
geneX transcript; mRNA
5′
3′
cDNA
C:DNA
B:RNA
A:DNA
5′
5′
3′
primer XF

Fig 2(2)

SAMPLE2; *geneX* transcript level '2'

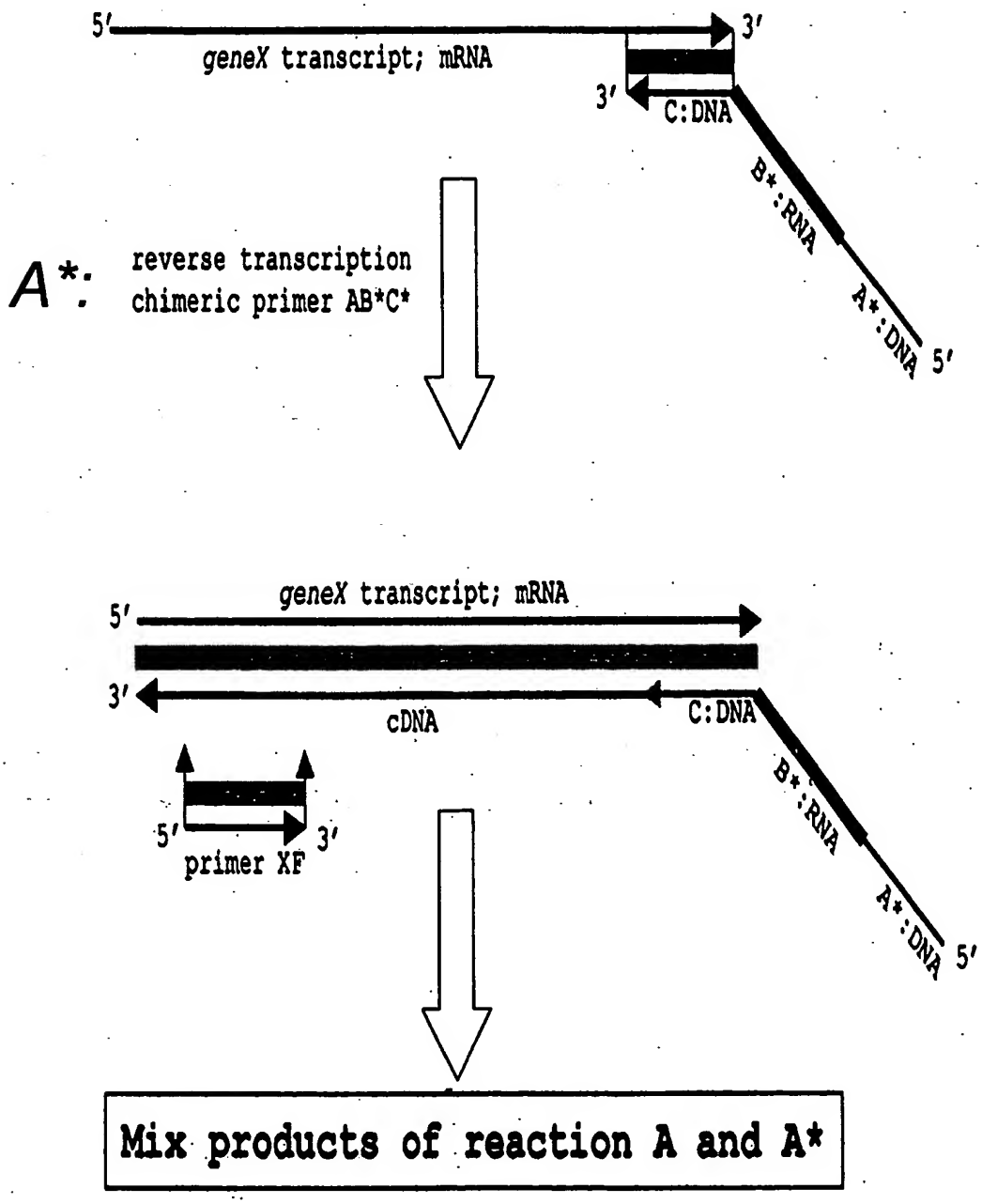


Fig 2(3)

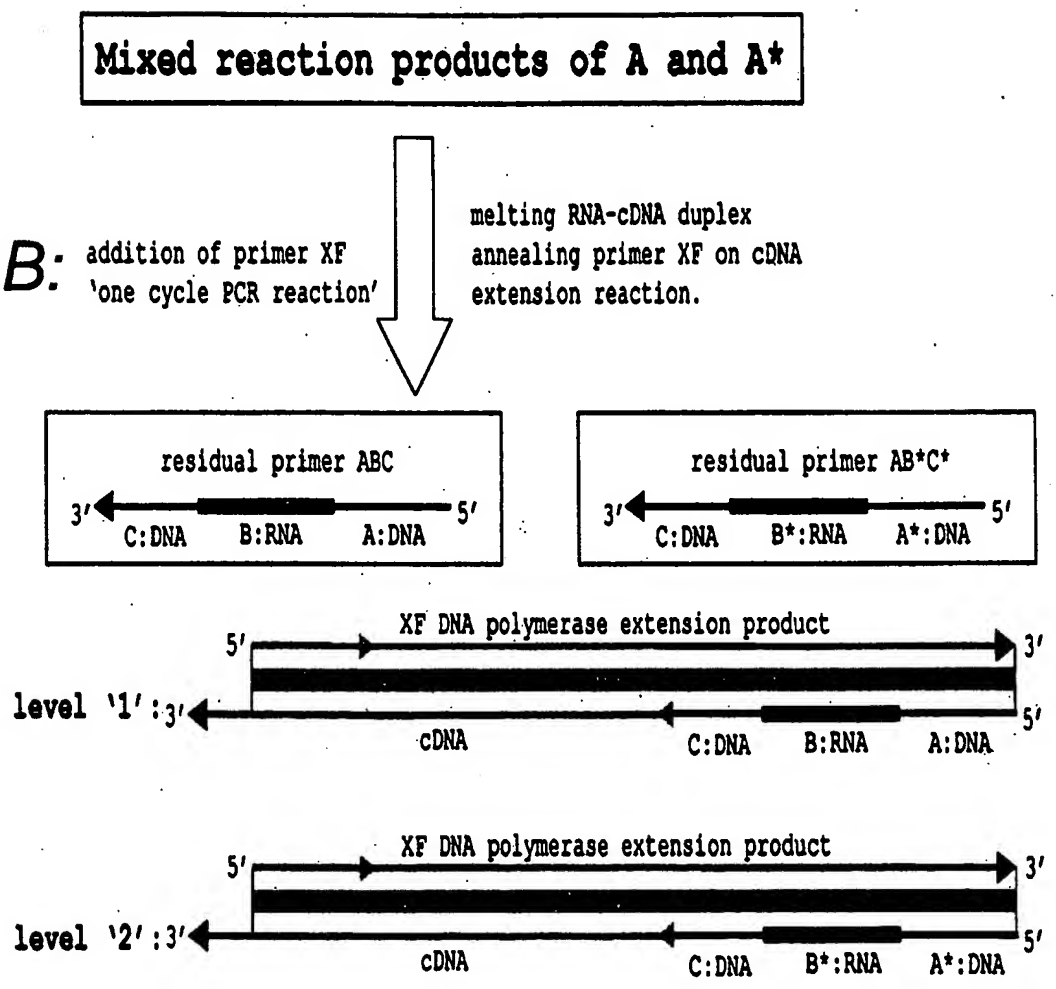

Mixed reaction products of A and A*
B.
addition of primer XF
'one cycle PCR reaction'
melting RNA-cDNA duplex
annealing primer XF on cDNA
extension reaction.
residual primer ABC
3'
5'
C:DNA
B:RNA
A:DNA
residual primer AB*C*
3'
5'
C:DNA
B*:RNA
A*:DNA
XF DNA polymerase extension product
5'
3'
level '1' :3'
5'
cDNA
C:DNA
B:RNA
A:DNA
XF DNA polymerase extension product
5'
3'
level '2' :3'
5'
cDNA
C:DNA
B*:RNA
A*:DNA

Fig 2(4)

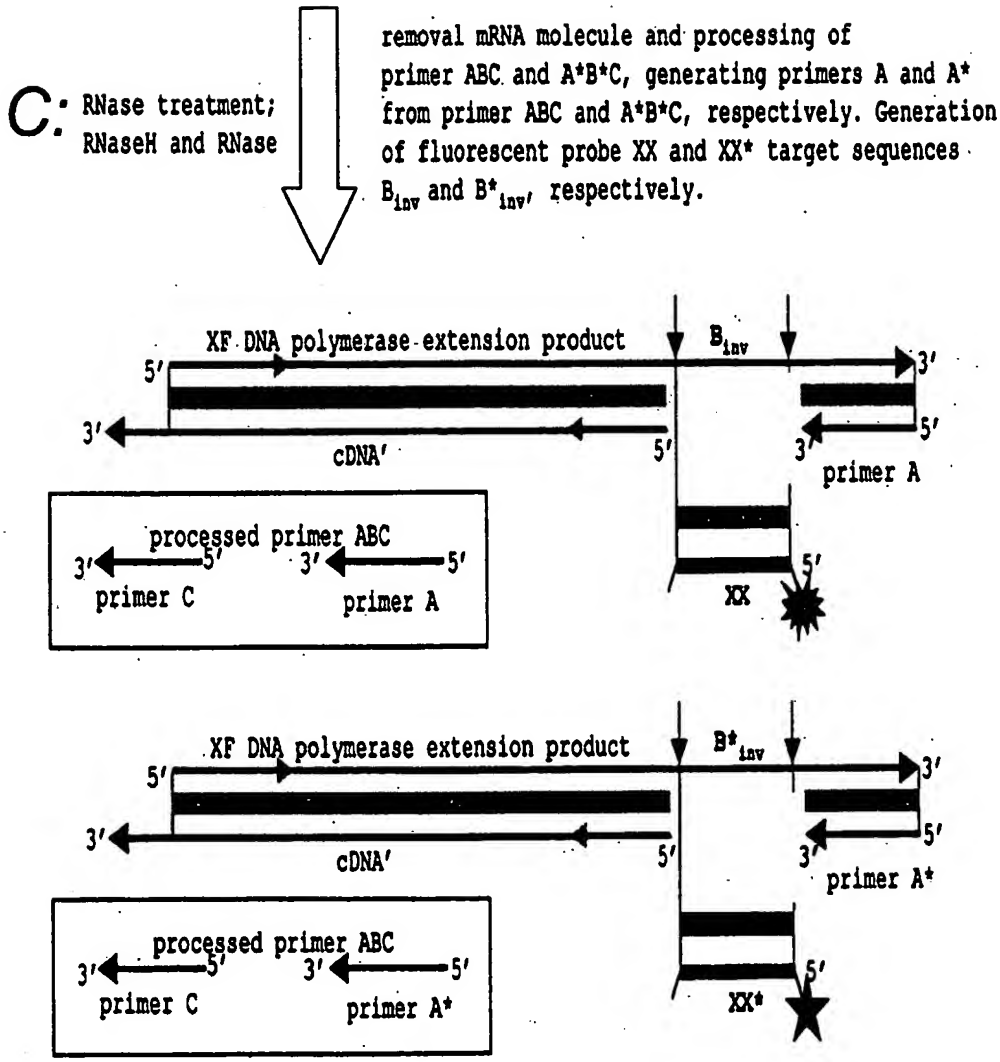

C: RNase treatment; RNaseH and RNase
removal mRNA molecule and processing of primer ABC and A*B*C, generating primers A and A* from primer ABC and A*B*C, respectively. Generation of fluorescent probe XX and XX* target sequences B_{inv} and B^*_{inv}, respectively.
XF DNA polymerase extension product
B_{inv}
5′
3′
cDNA′
primer A
processed primer ABC
primer C
primer A
XX
XF DNA polymerase extension product
B^*_{inv}
cDNA′
primer A*
processed primer ABC
primer C
primer A*
XX*

Fig 2(5)

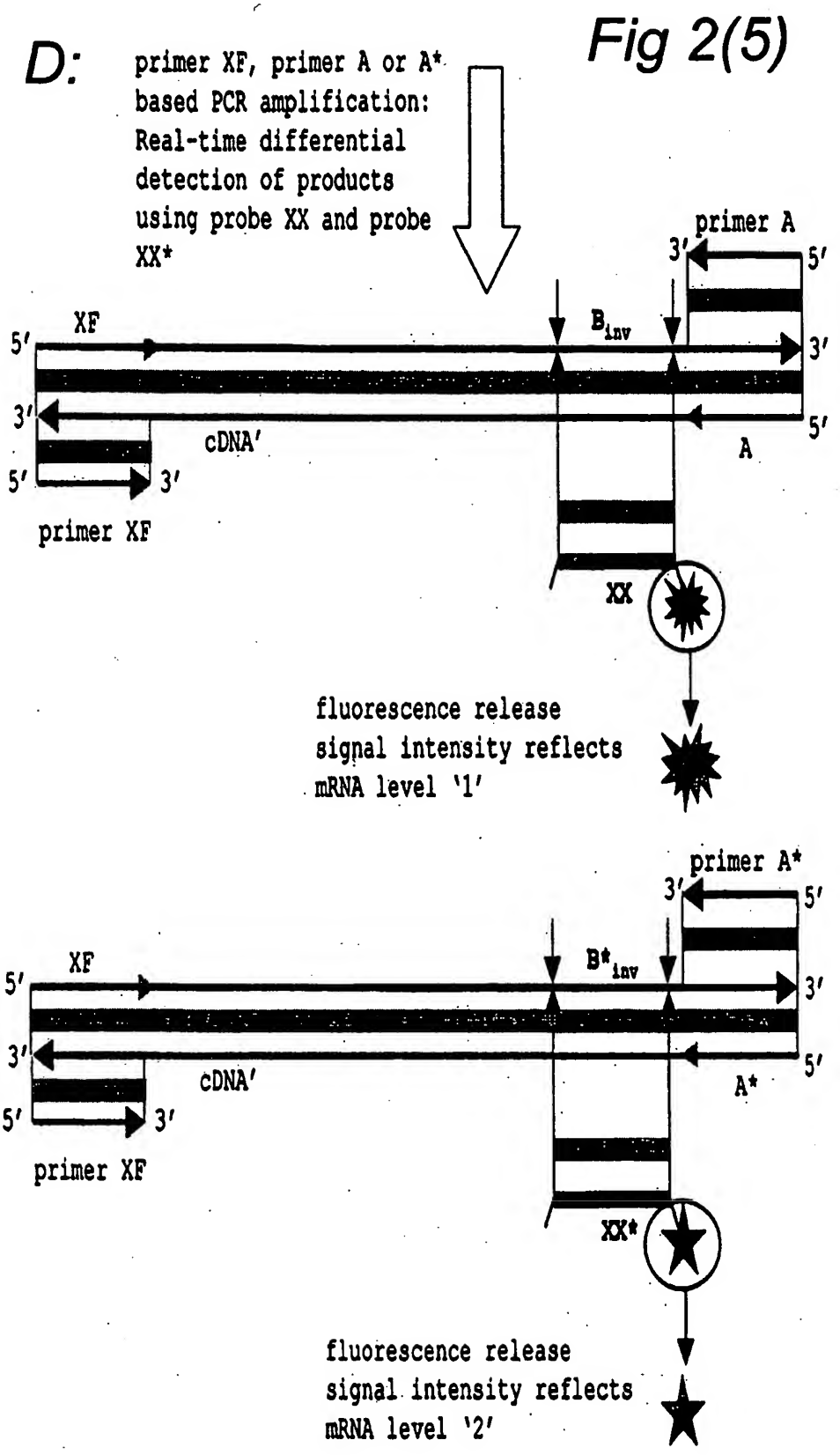
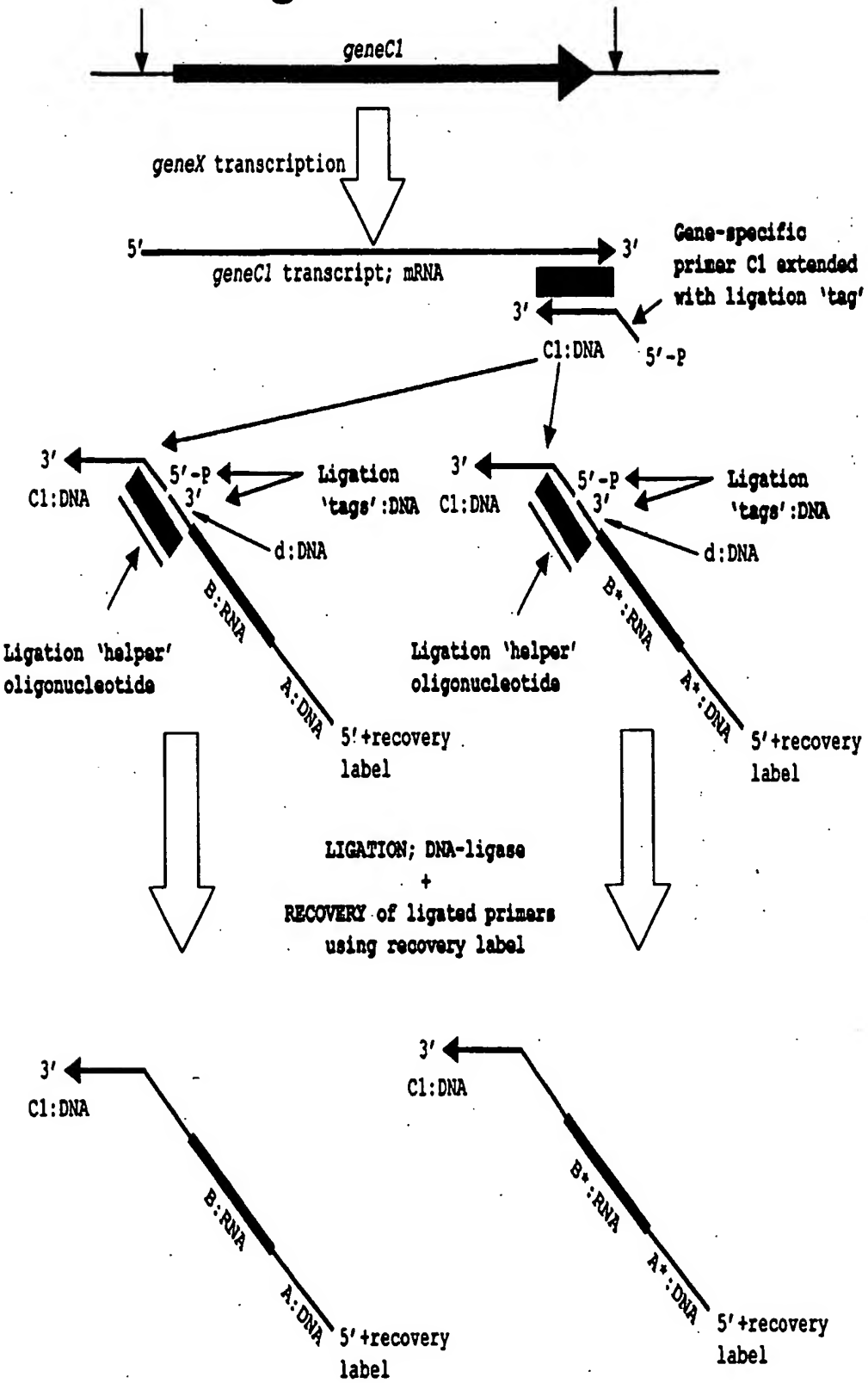

D:
primer XF, primer A or A* based PCR amplification: Real-time differential detection of products using probe XX and probe XX*
primer A
3′
5′
XF
5′
B_{inv}
3′
3′
5′
cDNA′
A
5′
3′
primer XF
XX
fluorescence release signal intensity reflects mRNA level '1'
primer A*
3′
5′
XF
5′
B^*_{inv}
3′
3′
5′
cDNA′
A*
5′
3′
primer XF
XX*
fluorescence release signal intensity reflects mRNA level '2'

Fig 3


geneC1
geneX transcription
5'
3'
geneC1 transcript; mRNA
Gene-specific primer C1 extended with ligation 'tag'
3'
C1:DNA
5'-P
3'
C1:DNA
5'-P
3'
Ligation 'tags':DNA
d:DNA
B:RNA
A:DNA
Ligation 'helper' oligonucleotide
5'+recovery label
3'
C1:DNA
5'-P
3'
Ligation 'tags':DNA
d:DNA
B*:RNA
A*:DNA
Ligation 'helper' oligonucleotide
5'+recovery label
LIGATION; DNA-ligase
+
RECOVERY of ligated primers using recovery label
3'
C1:DNA
B:RNA
A:DNA
5'+recovery label
3'
C1:DNA
B*:RNA
A*:DNA
5'+recovery label

INTERNATIONAL SEARCH REPORT

Ir tional Application No
I../NL 02/00519

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS, EPO-Internal, WPI Data, MEDLINE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 99 01571 A (MODRUSAN ZORA D ;ID BIOMEDICAL CORP (CA)) 14 January 1999 (1999-01-14) the whole document	1-23
A	US 6 251 600 B1 (WINGER EDWARD E ET AL) 26 June 2001 (2001-06-26) the whole document	1-23
A	EP 0 972 848 A (PERKIN ELMER CORP) 19 January 2000 (2000-01-19) the whole document	1-14

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

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Date of the actual completion of the international search	Date of mailing of the international search report
17 October 2002	07/11/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Ulbrecht, M

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL 02/00519

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HEATH KAREN E ET AL: "Universal primer quantitative fluorescent multiplex (UPQFM) PCR: A method to detect major and minor rearrangements of the low density lipoprotein receptor gene." JOURNAL OF MEDICAL GENETICS, vol. 37, no. 4, April 2000 (2000-04), pages 272-280, XP001106044 ISSN: 0022-2593 page 273, right-hand column, paragraph 5 -page 274, right-hand column, paragraph 1 figure 1	1-14

INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL 02/00519

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9901571 A	14-01-1999	AU 8327398 A	25-01-1999
		WO 9901571 A2	14-01-1999
		EP 0996743 A2	03-05-2000
		JP 2002507129 T	05-03-2002
		US 6274316 B1	14-08-2001
US 6251600 B1	26-06-2001	US 5853990 A	29-12-1998
		AU 3799497 A	20-02-1998
		CA 2262520 A1	05-02-1998
		CN 1236395 A	24-11-1999
		WO 9804738 A1	05-02-1998
EP 0972848 A	19-01-2000	US 5538848 A	23-07-1996
		EP 0972848 A2	19-01-2000
		AT 198775 T	15-02-2001
		AU 695561 B2	13-08-1998
		AU 4283696 A	06-06-1996
		CA 2201756 A1	23-05-1996
		DE 69519940 D1	22-02-2001
		DE 69519940 T2	23-05-2001
		EP 0792374 A1	03-09-1997
		JP 10510982 T	27-10-1998
		WO 9615270 A1	23-05-1996
		US 6030787 A	29-02-2000
		US 6258569 B1	10-07-2001
		US 5876930 A	02-03-1999
		US 5723591 A	03-03-1998